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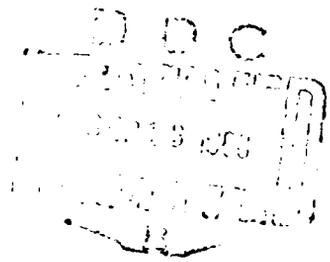

IMMUNITY OF WHITE MICE TO EEE-VIRUS

[Following is a translation of an article by Erich Traub of the West German Research Institute for Animal Virus Diseases in Tübingen, appearing in the German-language periodical, Zeitschrift für Immunitätsforschung (Journal of Immunity Research), Vol 122, 1961, pp 239-254.]

[This is the eighth report in a series on the above subject. The sub-title of this report is "Summarizing Discussion."]

Introduction

The view that immunity to animal virus diseases depended exclusively on the formation and action of specific antibodies has long held sway. Now we know that this immunity is instead, a complicated phenomenon. In the present state of research the following factors are held to play a part in that immunity:

1. The manner in which the cell reacts
2. The interference phenomenon
3. Specific and, in particular, neutralizing antibodies.

A certain amount of importance is attributed to phagocytosis in older works but no particular attention is any longer given it (6). In many cases it even has an infection-promoting effect because phagocytes are able to carry and spread virus particles among the tissues. Phagocytosis has therefore been omitted from our investigations.

Our experiments have been based on the working hypothesis that the proven mechanism of immunity found in

lysogenic bacteria was not renounced by Nature in the case of the higher phylogenetic orders as in mammals. Instead Nature merely improved upon it by adding a further safety factor, the antibodies. This accessory factor is however apt to disguise the effects of the basic factors.

The prophage in lysogenic bacteria (virus nucleic acid, genetic substance of the virus) is closely associated with the bacterial genome. It is non-infectious and does not multiply or reproduce itself independently. Instead it multiplies during the course of multiplication of the bacterial germ plasm (parasitism on the genetic level). The prophage behaves somewhat like a bacterial gene and is carried over to the daughter cells upon cell division. It retains its own characteristics, though, and in particular its capacity to break free of the bacterial genome under the influence of inducing factors (ultraviolet rays, X-rays, various chemicals) or to even develop spontaneously into pathogenic phage particles (vegetative phase) and to bring about lysis of the host cell. Upon completion of lysis infectious phage particles are liberated, infecting receptive bacteria and they can lead to the production of a new lysogenic system. Induction is not possible, however, in all lysogenic bacteria strains.

The prophage gives lysogenic bacteria immunity to the virulent form of the homologous phage and its mutants. These as a rule cannot reproduce themselves in lysogenic bacteria. This has been referred to as "intolerance of related phages on the prophage level." In rare cases, though, the infecting phage takes the place of the original prophages. Genetic recombinations can also occur. The bacterium cell loses its immunity upon induction. It can be superinfected with virulent mutants of the homologous phage during the vegetative phase. Usually lysogenic bacteria do not have any immunity to unrelated phages, but there are exceptions here, too.

For a bibliography on the subject and further details see the paper by Jacob and Wollman (12).

An attempt is made ~~in the following~~ discussion to sketch the mode of action and the significance of the various factors participating in active EEE (eastern equine encephalomyelitis) immunity in mice. ~~They are~~ dealt with in the same order in which they presumably come into play. The discussion includes the results of parallel investigations on lymphocytic choriomeningitis (LCM) in mice insofar as these findings contribute to those on EEE. In addition various points of view on the question of the "primitive" immunity mechanism of lysogenic bacteria are discussed.

Mode of Reaction of the Cells

The earliest investigations on LCM-immunity in mice gave the impression that immune substances in the body fluids played at least a very subordinate role and that the immunity is very closely bound up with the tissues which remain infected or have previously been infected (23). Rowe (18) came to the conclusion that cell reactivity appeared to be significant in this immunity. Experiments carried out by the author (34) have recently shown that a rapid multiplication of virus takes place during the first four days after in vitro infection in the lymph-node cells of adult mice which have not had any previous contact with the virus. This is followed by an equally rapid drop in the infectiousness titers of the cultures during the ten days which follow. The reproduction curve of the virus during the first six to seven weeks reminds one of a sine curve. Its shape gradually approaches a horizontal straight line upon prolonged culture. The reactivity of the cells in respect to the virus apparently undergoes a change with the passage of time. Further tissue culture experiments showed that a similar change in the mode of reaction of the lymph node cells in intolerant mice also took place in vivo as the result of an infection.

No similar tests had been carried out with EEE-virus. Mention should be made, though, of earlier observations of the multiplication of EEE-virus in the lung cells of normal mice [(39) See Tables 5 and 6]. When these observations were made no titration was carried out with periodically sampled culture fluids; they were tested intracranially in mice in undiluted form. One can nevertheless obtain an approximate picture of the virus content of the samples tested, judging by the incubation times and survival times of the individual experimental mice. The results of the infectiousness experiments enable one to conclude that the multiplication of virus in once tryptinized cells took place continuously and stopped only after the eighth day in the culture. On the other hand, in twice tryptinized cells the multiplication showed itself in most cases to be of a cyclical nature. This finding suggests that one can even artificially influence the mode of reaction of the cells. No attempt has yet been made to investigate how the lung cells of EEE-immune mice react to the virus in vitro.

Interference Phenomenon

Further investigations must be carried out before one can determine whether or to what extent the reactivity

of cells is associated with the interference phenomenon. According to Tyrrell (41) the cause of the wavy course of the hemag-glutinin curve in influenza-infected calf kidney cells is an alternating formation of virus and interferon (11, 11a) on the part of the infected cells. This can therefore also be assumed to apply to our system. Tyrrell, though, did not observe any change to take place over a three months period in the reactivity of the infected calf kidney cells, that is, he did not observe that the ryhthmic formation of interferons gradually became weaker and finally came almost entirely to a halt such as appears to be the case in the lymph node cells of mice (34).

The interference phenomenon as a factor in the immunity of LCM-immune mice.

The significance of interference as a factor in immunity in animals is perhaps best illustrated by the high level of cerebral immunity of mice congenitally infected with the LCM-virus. These mice are virus carriers throughout practically their entire lives and they continue to excrete virus, remaining immunologically tolerant, i.e., they do not form any specific antibodies (37, 27). Lymph node cells taken from such mice do not show any of the mentioned defensive reactions in cultures. They are therefore designated as tolerant (34). Tolerant mice could not be superinfected even with very large doses of LCM-virus (about 10^6 LD_{50}) (22, 29). Interference with the EEE-virus was also demonstrated to exist, but it was at least 30,000 times weaker than to the homologous virus (32).

Also in intolerant mice, which were immunized by subcutaneous injections of LCM-virus, followed by intracranial experimental infection, a degree of interference with the EEE-virus was found to exist similar to that found in tolerant animals (32). This interference continued to operate for at least six weeks, but dropped off greatly with the passage of time. Unlike the tolerant mice, these animals showed only in exceptional cases the presence of infectious LCM-virus in their brains and then only in traces up to the 21st day after intracranial reinfection with LCM. In keeping with the findings on tolerant mice one can assume that also in intolerant animals interference is very much greater and more lasting with the homologous virus than with heterologous EEE-virus. The strength of the homologous interference could not be measured in this case because it was necessary to also take into account the presence of specific antibodies, even if formed in only small quatities and hitherto found nowhere else but in the blood (18, 28). It must therefore be assumed that the high degree of cerebral

immunity shown by such mice depends almost exclusively on interference.

The part played by interference in active EEE-immunity in mice.

If one takes mice which previously had not become ill after subcutaneous infection with EEE-virus and subjects them to experimental intracranial infection with a large dose (about 10^6 LD_{50}) of the same virus, a large percentage of the animals usually becomes ill with encephalitis, ending in the death of part of the mice. Even in the case of the mice which did not become ill there was a multiplication of virus in the brain and in some cases it achieved considerable proportions. Regular tissue antibodies are not present at the time of the intracranial virus injection because in cases of abortive subcutaneous infection the central nervous system as a rule is not attacked (10, 25) and no antibodies are formed there (30). The circulating antibodies are insufficient to prevent multiplication of virus in the brain. It is true that local formation of antibodies takes place relatively quickly but it always lags behind multiplication of the virus (30). The absence of illness in the mice in spite of multiplication of virus in their brains must therefore have a different cause.

As we look on the underlying basic mechanism, it would seem to us that after intracranial injection the virus does not attack all the receptive cells in the brain simultaneously. This assumption is justified since both the number and extent of encephalitic lesions increase with time and also because after subcutaneous infection evidently not all the sensitive cells of the immunological system are infected simultaneously (See the following paragraph). The cells infected during the first cycle of infection and surviving the infection under the influence of the circulating antibodies go over into the interference stage and produce interferon instead of virus. It is probable that such a substance is involved even if no direct proof of it has been yet found. It is true that the interferon does not prevent infection of the remaining cells, but it inhibits the virus from multiplying in them. Such an explanation harmonizes with the findings of other authors using other systems (41, 42). There can be no doubt that the changeover into the interference stage under the influence of circulating antibodies actually takes place because encephalitis progresses very rapidly ending always in death in control mice having no body fluid immunity.

Time and again we were struck by the fact that both actively and passively immunized mice which had been experimentally infected intracranially and had afterwards

become ill but survived, showed a rather uniform set of symptoms. Partial paralysis of the forepaws severely affected the great majority and often persisted, sometimes disappearing with the passage of time. Such animals show a striking similarity to kangaroos in their attitudes and movements. This observation enables one to conclude that specific nerve cells in the central nervous system are either particularly susceptible to EEE-virus or they are harder to protect against the virus than other centers.

Proof of an interference phenomenon in actively immunized mice was achieved by reinfection with stepped doses of VEE (Venezuelan equine encephalomyelitis)-virus which is different from EEE-virus, but related to it (3). The heterologous interference was still unexpectedly powerful three weeks after intracranial experimental infection with EEE-virus (32). Here, too, by analogy with LCM, one can assume that the interference with respect to the homologous virus is still more powerful and lasting than with respect to a heterologous virus.

It should also be mentioned that mice which showed strong cerebral immunity to EEE-virus could not be shown to have any interference to the LCM-virus although a weak heterologous interference was regularly observed in the reverse direction (32).

The results of the interference experiments with the VEE-virus confirm earlier findings of a similar kind noted by Schlesinger, Olitsky and Morgan (19). Schlesinger (20) in a later paper, however, no longer attributed any importance to this earlier finding and explained the cerebral immunity in mice to WEE (western equine encephalomyelitis)-virus exclusively on the basis of the effects of antibodies, particularly those found in the tissues. We would prefer not to concur with this interpretation. In our opinion the high level of cerebral immunity lasting throughout the lifetime of those mice (36) surviving a first subcutaneous infection and then intracranial experimental infection with a large dose of EEE-virus depends on the combined action of both interference and specific antibodies, particularly those residing in the tissues.

The mechanism of the weak cerebral interference witt VEE-virus in mice not becoming ill after subcutaneous EEE-virus infection and not subjected to any subsequent experimental intracranial infection with the homologous virus still remains unexplained. Since a similar finding was made in principle in intolerant, LCM-immune mice (32), it is improbable that any antibody effect is present here.

One could imagine the possibility of involvement of an interferon having a remote effect on other organs.

Interference phenomenon after intracranial experimental infection of passively immunized mice.

Passively immune mice proved to be highly suitable test subjects. Hyperimmune homologous serum was injected into them intravenously. The antibody titer of the serums of such animals always remained higher immediately after treatment than in those mice which had achieved an active immunity after a subcutaneous infection which had run an abortive course (30, 31). After intracranial injection of small to average doses of EEE-virus there was a total neutralization of the virus in the passively immune animals and hence no illness. On the other hand intracranial injection of large quantities of virus brought about a delayed outbreak of encephalitis in a very high percentage of the mice with incubation periods of 11 to 76 days (26, 31). Such delayed outbreaks were first investigated in greater detail by Olitsky, Schlesinger and Morgan (16) in serum-treated, infected guinea pigs. Several of our test mice became ill with recurring encephalitis, the interval between first illness and recurrence of the disease varying between 12 and 70 days and averaging 34 days in eleven animals. Recurrent illness ended in death in all cases (33). The addition of antibodies suppressed the formation of self-produced antibodies, particularly in the central nervous system (24, 16, 26, 31). Also in cases of recurrent encephalitis the first attack did not induce the formation of any antibodies in the tissues (31).

The first assumption entertained, namely, that the injected immune serum brought about a delay in the outbreak of the encephalitis (26), has not been confirmed. Thus, it was found that intravenously injected immune serum disappears from the circulation within five to six weeks. Four to five weeks after injection one could find only slight traces of antibodies approaching zero in the serum. Upon recurrent illness after incubation periods of ten to eleven weeks the antibodies which had been added therefore could no longer have taken any effect. No substantiation could be found for any local formation of self-produced antibodies (31). It was therefore presumed that an interference phenomenon occurs in the brain in this case, too, and this can be substantiated by intracranial titration of VEE-virus during the latency period (33). It apparently takes place under the influence of the injected immune serum.

The virus remained in active form within the brain

during the artificially induced latency period (26). A renewed multiplication of the virus takes place to all appearances only after the interference has faded away. The uniformly mortal course of the recurrent illness permits one to conclude in any case that the animals had not received any antigenic stimulus as a result of the intracranial virus injection and the subsequent multiplication of virus in the central nervous system. If such had occurred then one would have been able to expect a recovery to occur in at least part of the animals after the renewed onset of virus multiplication.

Prevention of recurrence of the disease by virus injections during the latent period.

The successful prevention of recurrence of illness by repeated intraperitoneal injections of active virus during the period of latency and the failure of similar treatment with non-infectious formaldehyde-treated vaccine (31) lead one to think that the interference phenomenon has played a part here,too. In both cases there was a formation of circulating (high-titer) and tissue-located (relatively low-titer) antibodies. The antigenic effect was somewhat stronger in the former than in the latter case. This small difference cannot however be held solely responsible for the great difference in curative effects. On the other hand, it is difficult in this case to get a picture of the interference mechanism which in accordance with our experiments to date seems to be chiefly a local phenomenon. It is above all improbable that the virus succeeded in reaching the central nervous system in almost all the animals after interperitoneal injection, particularly so since the mice were of advanced age and the virus does not attack the central nervous system at all as often in them as in younger mice once peripheral infection has taken place. The question which arises at this point must therefore remain unanswered for the time being.

It would seem, on the other hand, that a pure interference phenomenon would lie at the basis of the prevention of recurrence of the disease by intracranial superinfection with VEE-virus during the stage of latency (33). Mutual exclusion of the participating viruses takes place, but the basic mechanism by which this occurs also remains as yet unexplained.

The Effect of Antibodies

Unlike the LCM-virus, the EEE-virus produces a powerful antigenic effect in mice. It is able to produce

hyperimmune sera, particularly if one hyperimmunizes the donor animals with virus material of the same kind (26).

Our investigations were limited chiefly to the effect of immune serum on the EEE-virus in infected cells in vivo and in vitro.

The effect of antibodies on intracellularly located virus antigens.

The suggestion that neutralizing antibody is able to take effect on the virus antigen located inside the cell has recently been disputed by the majority of virus researchers. The results of our tissue culture experiments (39) speak on behalf of the thesis that high-potency immune serum can irreversibly neutralize the virus in infected mice lung cells under optimum quantitative conditions, though we have not succeeded in regularly demonstrating this evidence to hold true for infected kidney cells.

Even if intracellular virus remains infectious in tissue cultures in the presence of immune serum, this does not unconditionally mean that the antibodies of the cells have not been assimilated. On one occasion the concentration of anti-bodies may not be great enough to completely neutralize the intracellular virus, while on another occasion some pure virus nucleic acid could be present along with whole virus elements inside the cell, the nucleic acid remaining unneutralized by the antibodies (1, 14, 21). This explanation is probably also applicable to recurring Herpes diseases in humans(9).

The findings in connection with human Lupus erythematosus (See bibliographies accompanying papers listed as references 15 and 13) leave hardly any doubt that antibody globulin (here we are dealing with pathological self-produced antibodies which chiefly damage the cell nucleus) succeeds in entering intact cells and carrying out its action inside them.

Inhibiting active immunization by injecting hyperimmune serum.

Serotherapeutic experiments carried out on EEE-infected guinea pigs (24, 16) have shown that active immunity often fails to be achieved even when the virus has already multiplied inside the organs before injection of immune serum. The strength of the sera used was found to be proportional to its inhibiting action in mice (26). Weak sera failed to interfere with the immunization; on the other hand, high-potency homologous immune serum prevented the development of active immunity in all animals when injected intravenously 48 hours after subcutaneous injection. By this time multiplication of virus had already long since

taken place in the organs (25) and the blood virus content which under existing conditions reaches its peak about nine hours after injection (35) was already dropping rapidly. The percentage of the mice achieving active immunity increased with the time interval between infection and serum injection, but a weak inhibiting effect could still be found present even after an interval of as long as seven days. A great many animals which had begun to produce antibodies of their own at the time of serum injection (4 to 6 days after infection) achieved only low serum titers. On the other hand, in the presence of fully developed active immunity the formation of antibodies on the 14th day after infection could neither be influenced by injection of immune serum nor by the organism's own antibodies produced during the intervening time. Accordingly, immunologically competent cells can no longer be inhibited from producing antibodies once such cells have received an antigenic stimulus. This finding permits one to conclude that part of the immunologically competent cells of the above mentioned mice showing only low serum titers had already received such a stimulus at the time of serum treatment.

The results argue in favor of the fact that the immune serum renders ineffective the virus antigens located inside the cells of the immunological apparatus. This would also still be held possible even if the cells had already started to produce their own antibodies. Apparently the antibodies, themselves, even contribute to this process. The loss of the virus's infectiousness decreased hand in hand with the removal of its antigenicity in the great majority of those mice which had been given the immune serum up to 72 hours after infection [(26), Table 9].

In accordance with these findings it appears improbable in the case before us that it is necessary for the virus antigen to persist in the cells in order that antibodies may go on being produced continuously.

The part played by antibodies in inactivation of virus in body organs.

The difference between actively immunized mice (30) and passively immunized mice (31) subjected to experimental infection with a large dose of EEE-virus is particularly impressive in this respect. An interference condition is brought about in the cells in both cases, but only the actively immunized mice form antibodies of their own in the central nervous system and the inactivation of virus in the brain occurs relatively more rapidly and is more lasting. It is hardly possible in this case to attribute this effect to any factor other than antibodies and here the tissue

antibodies are chiefly involved; this type of antibody does not form in the great majority of passively immune mice (31).

It was possible to achieve lasting inactivation of intracellular virus however, by heterologous interference even without the combined action of specific antibodies; in such cases there was a mutual exclusion of the participating viruses (33). Either of the two viruses, alone, would have killed the infected animal had it not been prevented from doing so by the presence of the other.

Anamnestic reaction.

Increased and accelerated formation of antibodies after reinfection can also be demonstrated to occur in EEE-immune mice (30), even if not as markedly as may occur when using many non-viral antigens. If one wishes to achieve a rapid local formation of antibodies in the brain and to strengthen cerebral immunity in actively immunized mice, this can only be done with very large doses of virus which are not rapidly neutralized by the antibodies put in circulation after the first infection. Such an injection leads to a practically absolute, long-lasting cerebral immunity.

It often happens that the animals give signs of encephalitic symptoms temporarily or even for extended periods after an abnormally short incubation period following intracranial injection (30). The cause of the shorter incubation period has not yet been explained. It could be a case of an antigen-antibody reaction or could be a cellular phenomenon. We suspect the latter, particularly in the previously described "accelerated reaction" after intracranial infection of LCM-immune (intolerant) mice with decreasing immunity (22, 18), because no evidence could be found of any local formation of antibodies in this case, even after intracranial virus injection. It is still uncertain whether this phenomenon has any causal relationship with the anamnestic reaction.

Immunity and Persistence of the Virus

The question as to whether the virus remains in the body in any form whatever upon postinfectional active immunity in men and animals is one of the most pressing problems in present-day virus research. Herriott (9) also attributes the same importance to this question and it is his opinion that free nucleic acid remains in infectious form in the cells of the immune animal. He looks upon a chronic infection limited in its extent as a means of maintaining a long-lasting immunity but he is also aware of the fact that the proof of any such lasting infection by viral nucleic

acid would be hard to demonstrate due to the presence of antibodies and nucleases.

We have failed to detect (40) infectious virus in the organs of EEE-immune mice after extensive experiments utilizing sensitive experimental methods (38). In no case did any hypothetical provirus become infectious such as not infrequently occurs with lysogenic bacteria (12). Unlike mammals, bacteria do not form antibodies and so far as is known to us, neither do they form any nucleases. Nevertheless, we believe that we should have been able to succeed at least once in detecting infectious virus or viral nucleic acid in the many infectiousness tests and tissue culture experiments if either of these had remained in the immune animals in the cases under consideration.

We had hoped to obtain some further proof either for or against the existence of a hidden prolonged or permanent infection by carrying out quantitative investigations of the changes in the antibody picture in mice which had been actively immunized with live virus, controls being injected with non-infectious formaldehyde-treated vaccine (36). These tests dealt with hemagglutination-inhibiting antibodies capable of being fairly accurately titrated without requiring a large expenditure of materials. In keeping with expectations the antibody titer of the serum always dropped more slowly in mice immunized with live virus than in vaccinated animals, although the latter as a rule showed higher titers at the start following intensive immunization. The difference was generally more marked in older than in younger mice. An antigenic stimulus appears generally to have a somewhat more lasting effect in younger mice, no matter whether live or dead virus is used for immunization (33). Many young mice immunized with live virus showed a rise in antibody titers lasting over a period of several months after infection and in some cases even continuing afterwards. Looked at as a whole, the results of the experiments aroused the suspicion that different immunity mechanisms were present in the mice immunized with live virus than in those immunized with formaldehyde-solution vaccine. The latter case presumably involves a pure antibody immunity while the post-infectional immunity appears to involve an additional factor which plays a part of its own.

In analogy to lysogenic bacteria one would also expect to find resistant cells in the immune animal in case of the existence of a provirus. Now, though, following Andrewes' outstanding investigations (2) of antiviral immunity, it is known that the washed cells of immune animals can be infected in vitro, that is, that they are not immune.

The question arises though as to whether this applies to all the live cells in the Maitland cultures used by Andrewes. It is stated elsewhere (39) that one must theoretically expect to find provirus-occupied cells alongside provirus-free cells in a culture of organ cells of an immune mouse, therefore one cannot assume that the EEE-virus infects all of the receptive organ cells in a mouse during the first stages of the virus or even later. This would already have to be considered improbable or unlikely in the light of the low infectiousness titer (25) which is usually higher upon infection of cells of the same organ in the tissue culture (38). Furthermore the lesions induced in the organ by EEE-virus have a focal character if any are to be found at all. In tests of our own (39) kidney cell cultures from highly immune mice were somewhat less receptive than those of normal animals. From a mathematical standpoint it was found in the first case that about 50% of the cells were resistant. Theoretically they could have contained a provirus.

It is impossible to exactly determine the relative importance of interference and of antibodies in actively immunized mice in vitro. The interference phenomenon undoubtedly plays a part in the beginning stage of immunity. Later, though, the antibodies cloud the true picture. Because of this we carried out parallel experiments with the LCM-virus which induces the formation of neutralizing antibodies to only a very limited extent in mice. The existence of a high degree of brain immunity in LCM-immune (intolerant) mice in whose central nervous systems neither active virus nor neutralizing antibodies could be detected (28) tends to substantiate the fact that one is dealing chiefly with an interference immunity in such cases. One could suspect the presence of a provirus as the chief interference-producing agent.

We hold it to be possible that the same immunity mechanism is present in men who suffer from agammaglobulinemia and who nevertheless achieve a normal immunity after infection with measles [Good and Zak, cited by Burnet (6)]. The reactivity of the cells presumably also plays a part in both cases.

Perhaps one may also be able to draw certain inferences about the circumstances found to exist in connection with EEE-immune mice on the basis of these findings.

Theoretical Considerations

We now visualize the immunity mechanism of those mice which failed to become ill after infection with EEE-virus as follows:

Presumably there is first of all immediately following the primary virus multiplication a cellular defense reaction similar to that which takes place in influenza-infected calf kidney cells (41) and in LCM-infected mouse lymph node cells (34). This goes on concurrently with the formation of interferon (41) which according to Isaacs et al (11a) inhibits the formation of viral nucleic acid. This can be used to explain the weak heterologous interference which has been demonstrated to occur in the brain after subcutaneous infection with EEE-virus (33) or LCM-virus (32). Possibly the behavior of the cells themselves is primarily responsible for the fact that the multiplication of virus in the organs takes place in only limited measure.

The immunologically competent cells in the mouse do not all receive the antigenic stimulus simultaneously but receive it instead in irregularly staggered time periods (39). Specific antibodies formed chiefly in the spleen and in the lymph nodes (25)can be detected in the serum from the third day onwards after infection (30). These antibodies inactivate the virus present in the infected cells of the immunological system during the days which follow (26, 39). The virus thereby loses its infectiousness and its antigenicity.

An interfering agent (provirus) which cannot be inactivated by antibodies nevertheless remains inside the infected cell. This provirus is non-infectious and represents a situation analogous to that of the prophages of lysogenic bacteria. It is carried over into the daughter cells and controls the production of antibodies on the part of the immunologically competent cells and their offspring. The cause of the immunity is to be found partially in the neutralizing effect of the circulating and stationary antibodies and partially in the interfering effect of this hypothetical provirus.

The interference effect of the provirus is presumably limited to those cells which contain the provirus. During the beginning stage of immunity an interferon is probably also involved inasmuch as the interference overlaps to affect also heterologous viruses. The strength of the immunity depends on the number of cells occupied by provirus. The immunity is at a high level in the central nervous system only if the virus has previously carried out an infection there (30, 36) giving the cells there a chance to become charged with provirus.

The fact that one succeeds in liberating both in vivo and in vitro infected cells of virus by treating them with hyperimmune serum (26, 39) permits one to suppose that

in the present case the antibodies are also able to neutralize the unattached viral nucleic acids which appear to be found in EEE-infected cells along with the integral virus (43). It is probable that such a reaction is possible because human Lupus erythematosus serum reacts serologically with pure desoxyribonucleic acid (See bibliographies accompanying works listed in references 15 and 13). In this case the latter works as a haptene, which can also be assumed to be true for ribonucleic acid. Nevertheless, a powerful effect can only be produced by hyperimmune serum taken from donor animals which have been repeatedly hyperimmunized; reconvalescent serum or immune serum from mice which have been given only one hyperimmunizing virus injection shows very limited effectiveness (26). We suspect that only very highly hyperimmunized serum is able to neutralize free viral-nucleic acid. On the contrary, it appears that after nucleic acid has been bonded with the genetic material of the cell (provirus) it can no longer be neutralized by even the most powerful immune serum (26).

The findings in connection with EEE-immune mice are more readily compatible with Burnet's clone-selection theory (5), which postulates a genetic carrying over of the information in the cells of the immunological system than with other hypotheses [Breinl and Haurowitz (4); Pauling (17); Günther (8), et al], which presuppose the persistence of virus antigens in the immunological system. This conclusion is based on the fact that antibodies make the virus antigen ineffective and that no basis has been given to date for the fact that the hypothetical provirus is able to become infectious each time and to form fresh virus antigen in EEE-immune mice (40). Even if this should be found to occur within limited areas without one being able to detect it due to the antagonistic effect of the antibodies and the ribonuclease (9), it would still be questionable whether it could effectively renew the immunity in the face of the inhibiting effect on immunization produced by the still-present antibodies.

The assumption that the provirus cannot again become infectious, as has been determined with non-inducible strains of lysogenic bacteria (12) or that its becoming infecticus has no practical effect on the immunity, makes it very hard to answer the question as to why the postinfectious immunity lasts longer than the immunity induced by inoculation with non-infectious formaldehyde-solution vaccine, even when in the latter case the original antibody titer of the serum was at first higher (36). It is difficult to answer this regardless of which theory of immunity one uses as a

basis. It is unlikely that the antibodies which come into being in the two cases are qualitatively different (7). One could presume by assuming a genetic theory that the genetic information transmitted in the two cases is in some way different. In taking a matrice theory as a basis one must assume that the antigen in the two cases persisted for different lengths of time in the immunologically competent cells.

The anamnestic reaction identifiable also in EEE-immune mice (30), the explanation of which places difficulties in the way of most immunity theories, can perhaps be correlated with cellular reactivity. As a necessary prerequisite to this it must first be found that the conclusions drawn from the LCM findings (34) are valid for other systems. When normal lymph node cells of adult mice first come in contact with the LCM-virus, allegorically expressed, they first take up a defensive attitude. They gradually become used to the virus, both in vitro and in vivo. This could be the reason why only a few antibodies are formed after the first injection of antigen while on the other hand a great many more are formed more quickly over a longer period of time after a second inoculation.

Arguments in Favor of the Existence of A Provirus

In conclusion we present below a list of reasons which in our opinion induce one to presume the existence of a provirus as a factor in immunity.

1. EEE: The active immunization can be prevented by treatment with high-potency immune serum after successful multiplication of virus in the organs even before receiving the antigenic stimulus (24, 16, 26). This is no longer possible after receiving the antigenic stimulus (26) although the added antibodies very probably also make those virus antigens to be found in the immunological system ineffective.

2. EEE: After immunization with live virus the production of antibodies continues for a longer period of time than after inoculation with non-infectious formaldehyde-solution vaccine, even when in the latter case the antibody titer of the serum was higher at first (36).

3. EEE: Trypsinized, washed kidney cells of hyper-immunized mice were somewhat less receptive to in vitro infection than the same type f cells from normal control animals (39).

4. EEE: Extensive investigations carried out with actively immunized mice failed to yield any evidence of infectious virus in spite of utilization of highly sensitive test methods (40).

5. EEE: An interference condition exists in the receptive cells of the central nervous system in both actively immunized (19, 30, 33) and passively immunized mice (31, 33) after intracranial experimental infection. It was impossible to determine the duration of its effectiveness as a factor in immunity in actively immunized mice because the antibodies located in the tissues disguise the picture.

6. LCM: Tolerant mice show a very high level, lifelong interference immunity in the absence of antibodies (22, 37, 27, 29). This shows that an immunity of this kind can be very effective. Such animals are nevertheless both virus carriers and secreters.

7. LCM: After subcutaneous first infection and intracranial reinfection, there exists in intolerant mice a high level of brain immunity (22, 18, 28) which also appears to depend primarily on the interference phenomenon, in spite of the absence of any proof of infectious virus and neutralizing antibodies in the tissues. The persistence of a provirus in such animals is presumably favored by a change in the reactivity of the cells after contact with the virus (34).

8. Measles: Human patients suffering from immunity subsequent to contracting the disease [Good and Zak, cited by Burnet (6)].

Summary

The experiments described in the seven preceding papers were based on the working hypothesis that a persisting provirus analogous to the prophage in lysogenic bacteria may be a fundamental factor in immunity to animal viruses and that the antibodies represent merely an additional even if often a highly effective safety factor apt to disguise the basic mechanism in many systems.

The discussion deals with the significance of cell reactivity, the interference phenomenon and neutralizing antibodies as immunity factors. Included are investigations made in parallel experiments with lymphocytic choriomeningitis (LCM) virus in mice as they supplement the results obtained with EEE-immune mice.

Cellular reactivity undoubtedly plays some part in the antiviral immunity of animals. It has not been studied extensively enough, though, to enable one to fully evaluate its role.

The part played by homologous interference is particularly evident from experiments on LCM in mice because antibody production is either absent or is much less intense than in EEE-immune mice. A state of interference arises in the infected cells of EEE-immune mice under the influence of antibodies. It is hard to evaluate this interference's importance as an immunity factor due to formation of local antibodies.

Much of the experimentation dealt with the in vivo and in vitro effects of neutralizing antibodies on intracellular virus. The results suggest that antibodies can destroy the virus's infectiousness and antigenicity but cannot reverse an antigenic stimulus. Persistence of viral antigen in immunologically competent cells does not appear to be a prerequisite to continued antibody production in EEE-immune mice. The observations agree with Burnet's clonal selection theory in this respect.

The following observations carried out on different viruses may be taken as indirect evidence for the persistence of a non-infectious but immunizing provirus in infected cells.

1. EEE: Above-mentioned effect of antibodies on viral antigen.

2. EEE: Antibody formation lasts longer in mice with post-infection immunity than in animals immunized intensively with non-infectious vaccine.

3. EEE: The slightly reduced susceptibility to in vitro infection of trypsinized and washed kidney cells from immune mice compared with the susceptibility of normal kidney cells.

4. EEE: The failure of extensive, sensitive tests to demonstrate existence of infectious virus in mice with post-infection immunity.

5. EEE: Interference in cerebral cells of cerebrally immune mice.

6. LCM: The remarkable effectiveness of a pure interference immunity without antibodies in congenitally infected, tolerant mice (virus carriers).

7. LCM: The "sterile" counterpart of this immunity in non-tolerant mice in which neither infectious virus nor antibodies in the tissues can be shown present in the brain.

8. Measles: Normal post-infection immunity acquired by patients suffering from agammaglobulinemia [Good, R. A. and Zak, S. K., Pediatrics, 18, 109 (1956)].

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